



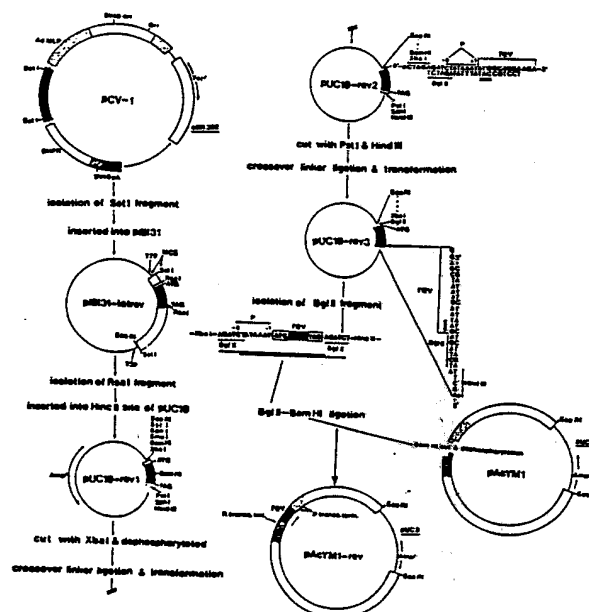
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(54) Title: IMPROVED BACULOVIRUS EXPRESSION SYSTEM CAPABLE OF PRODUCING FOREIGN GENE PROTEINS AT HIGH LEVELS

(57) Abstract

A baculovirus expression system capable of producing foreign gene proteins at high levels. The system involves the production of a recombinant baculovirus containing a modified foreign gene between the polyhedrin gene promoter region and the transcription termination signal of the polyhedrin structural gene. The modified foreign gene comprises a putative ribosome binding site immediately upstream of the foreign gene coding sequence, i.e. without any intervening non-coding sequences. The putative ribosome binding site is preferably properly positioned without the intervening sequences by a crossover linker mutagenesis procedure before the modified foreign gene is introduced into the virus. The putative ribosome binding site preferably comprises at least the final four nucleotides of the sequence ACCTATAAAT immediately upstream of the translation initiation codon (ATG) of the foreign gene. The system is capable of producing foreign gene proteins (when insect cells are infected with the recombinant virus) at high levels, even in the case of those genes which expressed only at low or intermediate levels in prior recombinant baculovirus systems.



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IMPROVED BACULOVIRUS EXPRESSION SYSTEM CAPABLE OF
PRODUCING FOREIGN GENE PROTEINS AT HIGH LEVELS

TECHNICAL FIELD

This invention relates to a method for increasing the yield of desired protein products obtainable by the expression of foreign genes in the baculovirus-cellular expression system using intermediate DNA modifications in the method, and to novel recombinant baculoviruses so-produced, particularly those which express HIV-1 specific rev, vif, pol and tat proteins upon infection of insect cells. The invention also relates to the utilization of these proteins for the development of prognostic reagents, diagnostic reagents and combined subunit vaccine against AIDS.

BACKGROUND ART

An important goal of recombinant DNA technology, as far as it relates to protein engineering, is to provide a gene expression system which will produce large quantities of desired gene products and provide protein modifications similar to those of the naturally occurring proteins.

Both prokaryotic and eukaryotic cells have been used to express cloned foreign genes and Escherichia coli is the most commonly used prokaryotic host system for foreign gene expression. However, prokaryotic cells are suitable for foreign gene expression only if the gene product does not require post-translational modifications such as glycosylation, phosphorylation or signal peptide cleavage. Since prokaryotic cells do not possess the appropriate machinery needed for the proper modification of many eukaryotic proteins, it has been necessary to develop gene expression systems using eukaryotes to obtain appropriately modified gene products. There have been impressive successes in the expression of foreign genes using eukaryotic hosts such as yeast, mammalian, plant and insect cells. The impetus for the development of new systems has come mainly from the need to produce larger

quantities of properly modified cloned gene products.

Advances in the genetics of invertebrate viruses and cells have allowed the development of viral-cellular systems which give both a high level of synthesis and complex processing of recombinant products. In particular, baculoviruses such as Autographa californica nucleopolyhedrosis virus (AcNPV) and Bombyx mori nucleopolyhedrosis virus (BmNPV) are extremely useful helper-independent eukaryotic expression vectors which are easily engineered. In the case of AcNPV, the system is based on a cell line established in the late 1970's from pural ovarian cells of the moth Spodoptera frugiperda. When infected with baculovirus carrying a foreign gene, these cells synthesize recombinant products complete with post translational modifications. In the case of BmNPV, foreign gene products can be expressed in living insects, namely silkworms. Both these viral systems are based on the utilization of the strong promoter of the gene encoding polyhedrin, the sole component of the crystalline matrix that acts as a protective shield for viral particles outside their insect host. The techniques conventionally employed in these systems are described in detail in U.S. Patent 4,745,051 to Gale E. Smith et al issued on May 17, 1988; Baculovirus Vectors for Expression of Foreign Genes, C. Yong Kang, Advances in Virus Research, Vol. 35, pp 177-192, Academic Press Inc., 1988; A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures, Max D. Summers and Gale E. Smith, May 1987, Texas A & M University; and Baculoviruses as Gene Expression Vectors, Lois K. Miller, Ann. Rev. Microbiol. 42, pp 177-199, 1988; the disclosures of which are incorporated herein by reference. This expression system has been used for the successful production of large quantities of many different gene products including human fibroblast interferon, human c-mye protein, human interleukin 2, etc. However, not all genes under the polyhedrin gene promoter express at high levels, e.g. those for HIV-1 specific rev, vif, pol and tat, as mentioned above. Many researchers who are

utilizing the baculovirus expression system have tried numerous techniques in order to improve the expression levels of such genes, but without much success (International Conference on Baculoviruses, Oxford, Great Britain, August 30 - September 3, 1988). Accordingly, the products which can be successfully produced by the system to date have been dependent upon the control mechanism that nature has selected for high level expression.

An object of the present invention is to provide a method of genetic engineering which provides high level expression of genes formerly expressing at only low or intermediate levels in the baculovirus-cellular expression system.

Another object of the invention is to provide refined site-directed mutagenesis methods with synthetic oligonucleotide linkers which can be used to engineer transfer vectors for the preparation of recombinant baculoviruses suitable for high level expression of foreign genes in the baculovirus-cellular expression system.

Yet another object of the invention is to provide recombinant baculoviruses capable of expressing desired foreign genes at a high level, particularly the human immunodeficiency virus genes pol, tat, vif and rev.

DISCLOSURE OF THE INVENTION

According to one aspect of the invention there is provided a recombinant baculovirus comprising at least a major part of a polyhedrin gene promoter region; at least a transcription termination sequence of a polyhedrin structural gene; a foreign structural gene having a translation start codon followed by coding sequences and a translation stop codon, said foreign gene being located between said promoter region and said termination sequence; and, immediately upstream of said start codon, at least a part of a putative insect cell ribosome binding site for the polyhedrin gene effective for at least partially overcoming resistance of susceptible insect cells to express said foreign gene at a high level.

According to another aspect of the invention there is provided a process for producing a recombinant baculovirus containing a foreign gene; said process comprising: providing said foreign gene having a translation start
5 codon followed by coding sequences and a translation stop codon; adding a nucleotide sequence immediately upstream of said start codon, said added nucleotide sequence comprising at least a part of a putative insect cell ribosome binding site for the polyhedrin gene effective
10 for at least partially overcoming resistance of susceptible insect cells to express said foreign gene at a high level; introducing said foreign gene and added nucleotide sequence into a baculovirus vector containing at least a major part of a polyhedrin gene promoter region
15 and at least the transcription termination sequence of a polyhedrin structural gene in a position and orientation to come under transcription control of said promoter region; cotransfecting susceptible insect cells with the resulting baculovirus vector DNA and wild type baculovirus
20 genomic DNA; and isolating recombinant viruses containing said foreign gene and added nucleotide sequence.

The invention also relates to recombinant baculovirus transfer vectors and processes for their production, proteins expressed by the novel recombinant baculoviruses
25 and uses of the proteins for medical reagents for medical tests and in vaccines.

The term "immediately upstream" as used above and throughout this disclosure means that there are no intervening nucleotides between the start codon (ATG) of the
30 foreign gene and the added putative ribosome binding site.

The term "a major part" of the polyhedrin gene promoter region means a sufficient part of the region to avoid loss of the effect of the promoter region during the transcription of the foreign gene.

The purpose of the invention is to increase the yield of proteins that would otherwise be expressed in low or intermediate yield in the baculovirus-cellular system. There is of course no great advantage in using the present invention to produce proteins that are already expressed in high yield. Although the terms "low", "intermediate" and "high" have not been formally defined in the art, in general it can perhaps be stated that when the desired protein forms less than about 1% of the total cellular protein the yield is considered to be low (and the protein is generally not visualized on polyacrylamide gel stained with Coomassie blue); a yield between about 1 and 10% of the total cellular protein is considered to be intermediate; and a yield above 10%, and preferably 15-50% or more, is considered to be high.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 is a schematic diagram showing a procedure according to a preferred embodiment of the invention in which a modified rev gene of HIV-1 is inserted into a pAcYM1 vector to form a transfer vector pAcYM1-rev suitable for forming a recombinant baculovirus AcNPV-HIVYKrev capable of producing rev at high levels;

Fig. 2 shows the time course results of a polyacrylamide gel electrophoresis of the proteins from cells infected with a recombinant AcNPV-HIVYKrev virus produced by the procedure of Fig. 1 and an AcNPV-HIVPKvif virus produced by the procedure of Fig. 3, showing the expression of rev and vif proteins;

Fig. 3 is a schematic diagram showing a preferred procedure for the modification of the vif gene of HIV-1 and its introduction into a pAcYM1 vector to form a transfer vector pAcYM1-vif suitable for forming a recombinant baculovirus AcNPV-HIVPKvif capable of producing vif at high levels;

Fig. 4 is a schematic diagram showing a preferred procedure for modification of the pol gene of HIV-1 and introduction of the modified gene into pAcYM1 transfer vector to form a transfer vector pAcYM1-pol suitable for forming a recombinant baculovirus AcNPV-HIVYKpol suitable for producing pol at high levels;

Fig. 5 shows the time course results of a polyacrylamide gel electrophoresis of total cellular proteins from cells infected with recombinant AcNPV-HIVYKpol virus; and

Fig. 6 is a Western blot of vif and rev proteins produced in accordance with Example 4.

BEST MODE FOR CARRYING OUT INVENTION

In the conventional baculovirus expression system, a foreign gene is inserted into the baculovirus genome as a partial or complete replacement for the polyhedrin structural gene while retaining the polyhedrin gene promoter and a stretch of the polyhedrin transcription termination signal. The promoter for the polyhedrin structural gene is allowed to remain so that it exerts a strong influence on the transcription but, as noted above, some gene products are nevertheless not produced at a high level.

The present invention is based on the introduction of a putative insect cell ribosome binding site immediately upstream of the foreign gene without intervening sequences under the polyhedrin gene promoter in the baculovirus transfer vector. In addition, the invention also involves the elimination of any non-coding flanking sequences at preferably both the 3' and 5' ends of the foreign gene using a uniquely modified crossover linker mutagenesis method. This modification of the baculoviral vector overcomes any tendency of the viral-cellular system to resist expression of the foreign gene.

The putative insect cell ribosome binding site referred to in the present invention is the sequence of up to 10 bases immediately upstream of the translation initiation site (ATG) of the natural polyhedrin structural gene, i.e. the underlined nucleotides in the sequence:

```

              -50  TRANSCRIPTION INITIATION
              |
(5')  .... TAAATAAGTATTTTACTGTTTTCGT
        polyhedrin promoter
        AACAGTTTGTAAATAAAAAACCTATAAAT  polyhedrin
        ..... ATG ..... (3')
              |           |
        Putative Ribosome Binding Site  Start Codone
  
```


The sequence is referred to herein as the "putative" ribosome binding site because there has been as yet no experimental verification that this sequence, when transcribed, takes part in ribosome binding.

5 While this sequence is present immediately upstream of the polyhedrin structural gene in wild type baculoviruses, the sequence is partially eliminated and/or displaced upstream of the start codons of foreign genes inserted into known baculovirus transfer vectors. It has now been found
10 that the introduction of the putative ribosome binding site immediately upstream of the foreign gene start codon without intervening flanking sequences and/or restriction enzyme sites overcomes any resistance of the cell to express the foreign gene at only low or intermediate levels. The entire
15 putative ribosome binding site sequence need not be introduced and instead merely a part of the sequence that is effective to improve expression yields can be introduced. The final part of the sequence appears to be the most important and must normally be present. It is believed that
20 as few as the final four nucleotides, 5'-AAAT-3', can improve expression yields, but at least the final eight nucleotides, 5'-CTATAAAT-3', are normally provided. More preferably, the added sequence contains the nine nucleotides (5'-CCTATAAAT-3').

25 As described in more detail below, the above sequences are most conveniently introduced, and non-coding sequences flanking the foreign gene are most conveniently eliminated, by means of a crossover linker mutagenesis strategy employing single stranded, or more preferably double stranded,
30 oligonucleotide linkers. Furthermore, the same strategy is normally used to remove any non-coding flanking sequences at the 3'-end of the foreign gene and to add a restriction site at this end.

35 In general terms, the crossover linker mutagenesis procedure can be described as follows. The foreign gene is synthesized or isolated from a suitable DNA or RNA source (e.g. a commercially available plasmid having suitable

restriction sites bracketing the foreign gene) and is inserted into a small plasmid using standard techniques. If isolated from a natural source, the gene is normally accompanied by non-coding flanking sequences and, to the extent possible, these are partially removed by standard digestion and ligation techniques.

A suitable oligonucleotide linker for upstream modification of the gene is produced using standard DNA synthesizing techniques. This linker may be single stranded, but is more preferably double stranded, especially if it is desired to introduce a restriction enzyme site in the linker. If a single stranded linker contains a restriction site, the efficiency of crossover mutation drops because of self annealing of the self complementary palindrome sequences. The linker, or the primary strand if a double stranded linker is employed, normally contains a sticky end restriction site and a different restriction site, e.g. Bam HI or Bgl II, immediately upstream of (and possibly partially overlapping) the effective putative ribosome binding site sequence, followed by at least 9 and preferably 12-15 bases of homology searching sequences which represent the first NH₂-terminal 4-5 amino acids coding sequence of the foreign gene. It is important to avoid, if possible, any homopolymeric sequences in the homology searching sequences since some DNA molecules contain a stretch of homopolymer. When the linker is double stranded, the second strand comprises the complementary sequence except for the missing bases necessary to form the sticky end restriction site and for three to five missing bases at the opposite end to form a single stranded overhang (the latter being necessary to avoid blunt end ligation of the linkers during the crossover mutagenesis).

The plasmid containing the foreign gene is linearized using a restriction endonuclease digestion which acts on a restriction site upstream of the foreign gene and the ends of the linearized plasmid are preferably dephosphorylated to prevent re-circularization. Alternatively, two restriction endonuclease digestions can be used to avoid recircularization. The oligonucleotide linker is ligated by virtue of

its sticky end restriction site to the linearized plasmid and the resulting modified structure is introduced into a suitable competent cell system, preferably E. coli, by the standard DNA transfection method. The transfected cells are
5 capable of deleting unwanted bases flanking the foreign gene and circularizing the plasmid.

A restriction site is also normally introduced at the 3' end of the foreign gene and any unwanted non-coding sequences at the 3' end are preferably deleted by a similar crossover
10 linker mutagenesis technique using a single or double stranded linker. In this case, the linker comprises a minimum of 9 to 12 bases of homology searching sequences corresponding to the final coding sequence of the foreign gene at the 3' end, followed by the restriction site and a
15 sticky end of a different restriction site. The plasmid containing the modified foreign gene resulting from the previous crossover linker mutagenesis is then linearized at a site downstream of the 3' end of the foreign gene, the oligonucleotide linker is ligated and the resulting DNA
20 structure is transfected into a competent microorganism, again preferably E. Coli, which deletes the unwanted flanking sequences, adds a desired restriction enzyme site and recircularizes the plasmid.

The modified foreign gene can then be cut out and
25 inserted into a baculovirus transfer vector from which part or all of the polyhedrin structural gene has been excised and which contains a suitable cloning site downstream of the transcription initiation site of the polyhedrin promoter region of the vector. Since various baculovirus transfer
30 vectors containing suitable cloning sites are readily available, it is advantageous to start with such a known vector rather than construct a new one specifically for this invention, although this could be done if desired. The baculovirus transfer vector employed should preferably have
35 an intact polyhedrin promoter region (e.g. pAcYM1 or pVL941) but those with partial deletions may also be employed,

provided they are still capable of high level transcription. For example, vectors pAc373, pAcRP6 and pAc610, which start at the -8 position of the upstream sequences, can be employed (see the article by C. Yong Kang mentioned
5 above). The two most efficient transfer vectors appear to be pACYM1 and pBM030 (available from Drs. Bishop in England and Maeda in Japan, respectively) which contain all of the upstream sequences of the polyhedrin gene adjacent to a Bam HI restriction site (pACYM1) or a Bgl II
10 restriction site (pBM030). The baculovirus transfer vector should also contain the transcription termination codon and preferably the polyadenylation sequences of the polyhedrin gene. The vectors are linearized by appropriate restriction endonuclease digestion followed by phosphatase
15 treatment.

The foreign gene having the modified flanking regions is inserted into the restriction site of the baculovirus transfer vector and the orientation of the foreign DNA insert is then determined by standard restriction
20 endonuclease mapping and/or DNA sequencing. The resulting baculovirus transfer vector containing the modified foreign DNA is amplified and purified by standard techniques.

After the foreign gene with the desired upstream
25 putative ribosome binding sequences has been inserted into the transfer vector, the construct DNA is cotransfected into suitable insect cells with purified authentic wild type baculovirus DNA of the same strain, e.g. by the procedure as outlined in U.S. Patent 4,745,051 mentioned
30 above. The insect cells are generally employed as a monolayer and, following infection, are incubated in a suitable culture medium for a number of days and the supernatant is harvested. Polyhedrin-negative viruses resulting from homologous recombination appear as clear
35 plaques in plaque assay and can be selected by plaque picking. An alternative approach to this biological assay system is to screen polyhedrin-negative plaques by nucleic

acid hybridization techniques using the cloned foreign DNA as a hybridization probe.

The recombinant virus can then be propagated after successive plaque isolation to exclude wild type viruses
5 by isolating a single plaque and amplifying the virus in monolayer culture in a suitable culture medium. After a few days of infection, the supernatant can be harvested and used to infect large numbers of cells in suspension or monolayer cultures.

10 The resulting recombinant virus, which forms a vector for the expression of the foreign gene, can be used to infect appropriate insect cells or insects, whereupon the gene is expressed and the desired protein forms in high yield. If the gene product is a secretory protein such as
15 IFN, IL-2 or HBsAg, the infected cells release these proteins after synthesis and these can be recovered from the extra cellular fluid of cultured cells or from the hemolymph of the infected insects. In contrast, if the protein in nature is phosphorylated and anchored in the
20 cell, the expressed gene products remain in the infected cells and can be recovered from the cells after 2-4 days of infection. For example, the pol, tat and rev proteins of HIV-1 remain in the nucleus whereas human hepatitis B virus surface antigen (Kang et. al., J. Gen. Virol. 68:
25 2607-2613, 1987) and gp 120 of HIV-1 (Bishop, Oxford, UK - Personal Communication) are secreted into the extra cellular culture fluid.

The expressed gene products may be analyzed by direct protein analysis using polyacrylamide gel electrophoresis
30 and Coomassie blue staining.

As noted above, the method of the invention can be used with a variety of baculovirus-cellular systems, the preferred ones being Autographa californica nuclear polyhedrosis (particularly the H1 strain used in the later
35 Examples) which infects Spodoptera frugiperda cells, and Bombyx mori which infect silkworm cells. (However, virtually any species or strain of baculovirus may be

employed). Other viruses and strains include those listed in U.S. Patent 4,745,051 (col. 9, lines 21-39).

The preferred restriction site introduced with the putative ribosomal binding site is Bam HI, but other
5 restriction sites which provide compatible cohesive ends (i.e. isoenzyme sites) can be employed, e.g. Bcl I, Bgl II, Mbo I and Xho II which all produce the 5'-GATC-3' sequence upon digestion, as does Bam HI itself. The possibility of using alternative restriction sites is
10 convenient when the foreign gene DNA itself contains internal Bam HI or Bgl II restriction sites.

Instead of using the crossover linker mutagenesis strategy for modifying the foreign gene prior to its introduction into the baculovirus transfer vector, it
15 would be possible to achieve the same results by ligation of the linkers to the linearized gene-containing plasmid after deletion of the non-coding flanking sequences by exonuclease digestion e.g. with Bal 31. However, such a technique is very imprecise and difficult and, while
20 included within the scope of the present invention, is not the preferred technique.

The present invention makes it possible to produce proteins at high levels of expression and many of these proteins can then be used for medical purposes such as for
25 prognostic reagents, diagnostic reagents and combined subunit vaccines. The rev, vif, pol and tat proteins of HIV-1 produced in this way are particularly useful for the management of acquired immunodeficiency syndrome (AIDS), e.g. by the techniques indicated in the publication
30 entitled Clinica, Testing for HIV and AIDS, The Next Five Years, George Street Publications Ltd., Richmond, Surrey, UK, the disclosure of which is incorporated herein by reference.

A particularly useful diagnostic or prognostic test
35 involves the use of the vif protein. The vif protein is one of the regulatory proteins of HIV-1, but the body produces antibodies against this protein as well as

antibodies against the virion structural proteins (gag, pol and env). It has previously been indicated that putative vif protein, expressed in bacteria, react with sera from HIV-1 infected individuals at all clinical stages (Kan, N.C. et al, Science 1986, 231, 1553-1555). However, in contrast to such previous reports it has now been shown that antibodies against full length vif proteins expressed in Spodoptera frugiperda (SF9) infected with recombinant baculoviruses appear early in HIV-1 infection and disappear as clinical signs of AIDS appear, whereas antibodies against rev protein (a transregulatory gene product) are present randomly in all clinical stages of HIV-1 infection. Consequently, vif protein may be used as a prognostic indicator for the development of AIDS.

The differences between the previous reports and the findings of the present invention may be due to the source of the vif protein. The previous studies used antigens expressed in bacteria which show a high degree of non-specific cross-reactivity, whereas vif proteins expressed in insect cells show a clean background in Western blots.

Presently preferred embodiments of the present invention are described in the following Examples.

These Examples relate to the preparation of the rev, vif and pol proteins of HIV-1. However, the tat protein of HIV-1 has also been produced by similar techniques. The recombinant baculovirus capable of producing the tat protein (AcNPV-tatYK) was deposited on March 4, 1988 at the American Type Culture Collection of 12301 Parklawn Drive, Rockville, MD 20852, USA under the terms of the Budapest Treaty and the deposit is identified by the number ATCC VR 2206.

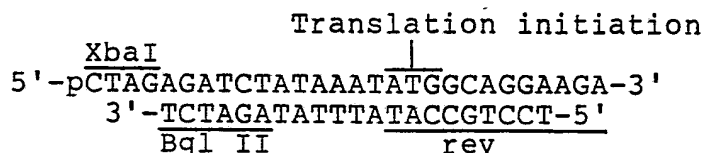
EXAMPLE 1 PRODUCTION OF THE rev PROTEIN OF HIV-1.

A recombinant baculovirus containing the rev structural gene and the additional sequences required by the present invention was produced by a procedure as shown in Fig. 1.

The coding sequences of the rev protein were originally isolated from the Sst 1 fragment of pCV-1 plasmid. The Sst 1 fragment was inserted into pIBI31 plasmid. The Rsa1 fragment

containing the rev coding sequences was isolated and inserted into the Hinc II site of pUC19. The resulting pUC19-rev 1 plasmid was then digested with Xba I and dephosphorylated and a double-stranded crossover linker was ligated to the Xba I site of the linearized pUC19-rev 1 plasmid.

A double stranded crossover linker was synthesized using standard DNA synthesizing techniques. The first linker strand comprised an XbaI sequence suitable as a sticky end (CTAGA), a Bgl II restriction site (AGATCT) (this restriction site is used because rev gene contains an internal Bam HI site), a TATAAAT sequence, and the initial 12 nucleotides of the coding sequence of rev (ATGGCAGGAAGA). The second linker strand comprised the complementary sequences of the first linker strand but omitting the sequences at one end required to form the Xba I sticky end and omitting the final 3 nucleotides at the opposite end to form a single stranded tail. The linker strands were then annealed to form the following double stranded linker:

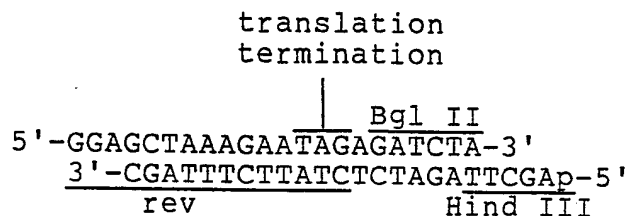


The double-stranded crossover linker was ligated to the Xba I site of the linearized pUC19-rev 1 plasmid and the resulting elongated linearized recombinant plasmid was transfected into competent *E. coli* cells to carry out a crossover linker mutagenesis. Ampicillin resistant cells were selected and cloned, and the resulting pUC19-rev 2 plasmids containing transformants were isolated.

The plasmid pUC19-rev 2 contained the desired sequence upstream of the rev gene but also contained unwanted non-coding sequences downstream of the rev gene and these were removed by the following technique:

A second double stranded oligonucleotide linker was synthesized by a standard DNA synthesis technique. The first strand of this linker comprised the final 15 nucleotide sequence of the rev gene including the translation

termination codon, a Bgl II site and a nucleotide for a Hind III site. The second strand comprised the Hind III sticky end and the complementary sequences of the first strand, except for the final three nucleotides. When
 5 annealed, the double stranded linker thus was as follows:



The pUC19-rev 2 DNA was cut with Pst I and Hind III without dephosphorylation, the second double stranded synthetic linker was ligated to the Hind III site and the
 10 resulting elongated linearized plasmid was transfected into competent E. coli cells. The ampicillin resistant cells were selected and cloned. The bacterium recircul-
 arized the plasmid and deleted the unwanted downstream sequences to form plasmid pUC19-rev 3. This contained
 15 XbaI followed by Bgl II, CTATAAAT (partially overlapping the Bgl II site and forming the putative ribosome binding site of S. frugiperda cells) and the entire coding
 sequence of rev followed by Bgl II and Hind III.

The rev gene-containing sequence was isolated using
 20 Bgl II digestion and was ligated into a baculoviral transfer vector pACYM1 that had been linearized with Bam HI and dephosphorylated, to give a desired vector pACYM1-rev.

The vector was then used to cotransfect Spodoptera
 25 frugiperda cells together with wild type AcNPV DNA and polyhedrin-negative recombinant viruses AcNPV-HIVYKrev were selected and amplified.

AcNPV-HIVYKrev was used to infect S. frugiperda cells which were harvested 24, 48, 72 and 96 hours after
 30 infection and the recombinant virus infected cellular proteins were subjected to protein analysis by poly-
 acrylamide gel electrophoresis with Coomassie blue staining as shown in Fig. 2. In the Figure, lane 1 shows

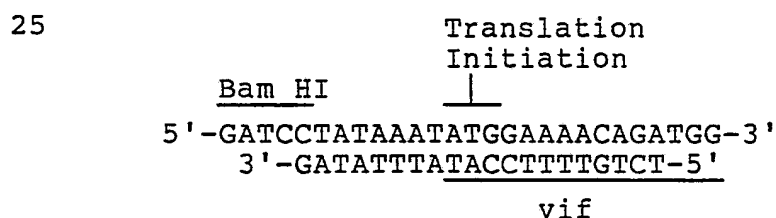
uninfected S. frugiperda cells, lane 2 shows wild type AcNPV infected cells, lane 3 shows AcNPV-HIVYKrev virus infected cells and lane 4 shows AcNPV-HIVPKvif virus infected cells (pertinent to Example 2). The symbol p denotes the polyhedrin protein, v denotes vif protein and r denotes rev protein. The M lane shows molecular weight markers. A band representing the rev protein is clearly visible indicating a large yield (ca 20%) of this protein.

The recombinant virus AcNPV-HIVYKrev was deposited at the American Type Culture Collection of 12301 Parklawn Drive, Rockville, MD 20852, USA on November 30, 1988 under the terms of the Budapest Treaty and the deposit is identified by the number ATCC VR 2231.

EXAMPLE 2 PRODUCTION OF vif PROTEIN OF HIV-1

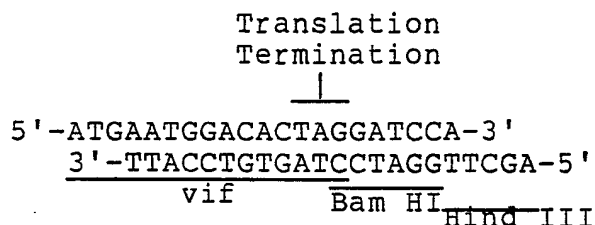
As shown in Figure 3, using techniques similar to those of Example 1, the vif gene containing the entire coding sequences was isolated from the plasmid pHXB-2D by EcoRI digestion (the coding sequence of vif is located within the EcoRI fragment-mapping unit of 4227-5322 bps and approximately 1100 bps were isolated). The EcoRI fragment was filled in with Klenow and inserted into the Hinc II site of pUC19 (pUC19-vif 1).

Using standard DNA synthesizing techniques, the following double stranded linker was synthesized;



This linker contained a Bam HI sticky end at the 5' end followed with a CCTATAAAT sequence (the putative ribosome binding site p) with 12 nucleotide coding sequences of the vif gene. This double stranded linker was used to modify the upstream sequences of the vif gene by cutting the plasmid pUC19-vif 1 with BAM HI and XbaI, ligating the linker and transforming competent E. coli cells as in Example 1. This resulted in the formation of a recombinant plasmid pUC19-vif 2.

To modify the downstream sequences, the following oligonucleotide linker was synthesized;



This linker contained a 5'-12 nucleotide overlapping sequences of the vif gene which included the translation-termination signal TAG followed by Bam HI and Hind III sticky end. The 22 nucleotide long complementary sequence with a Hind III sticky end was used to protect the Bam HI site.

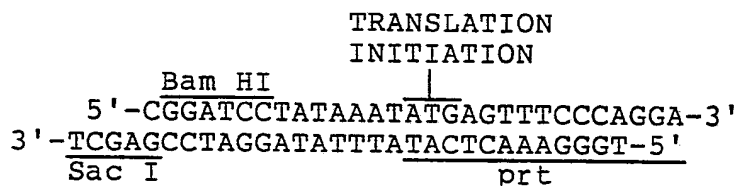
The recombinant plasmid pUC19-vif 2 was cut with Pst I and Hind III, the double stranded linker was ligated at the Hind III site and the plasmid was used to transform competent E. coli cells. This resulted in the deletion of the 3' non-coding sequences of the vif gene and the addition of a Bam HI restriction site. The resulting pUC19-vif 3 plasmid contained Bam HI sites at either the putative ribosome binding site (p), the entire coding sequence of vif including the translation termination coding sequence TAG at the end. This Bam HI fragment was isolated and inserted into the Bam HI site of pAcYM1 in the correct orientation (pAcYM1-vif). The pAcYM1-vif DNA was used to transfect Spodoptera frugiperda cells with wild type AcNPV DNA to isolate the recombinant baculovirus AcNPV-HIVPKvif. The recombinant baculovirus, AcNPV-HIVPKvif, was used to infect Spodoptera frugiperda cells to express the vif gene. The AcNPV-HIVPKvif virus infected cells produced a 26K Dalton protein (v) which represents at least 30% of the total cellular protein at 96 hours after infection, as shown in Figure 2.

The AcNPV-HIVPKvif virus was deposited at The American Type Culture Collection of 12301 Parklawn Drive, Rockville, MD 20852, USA on February 15, 1989 under the terms of the Budapest Treaty and the deposit is identified by the number ATCC VR 2235.

EXAMPLE 3 PRODUCTION OF THE pol PROTEIN OF HIV-1

As shown in Fig. 4, using techniques similar to those of Examples 1 and 2, the pol gene-containing part of the protease gene at the 5' end followed by the entire coding sequence of the reverse transcriptase gene and the coding sequences of the integrase at the 3' end were isolated from a plasmid pHXB-2D by digesting the plasmid with Bgl II and Sal I. The Bgl II-Sal I fragment was then inserted into plasmid pUC18 and upstream and downstream sequences were modified to remove some of the non-coding flanking sequences. The pUC18 containing the entire coding sequences of the polymerase gene was digested with Sac I and dephosphorylated.

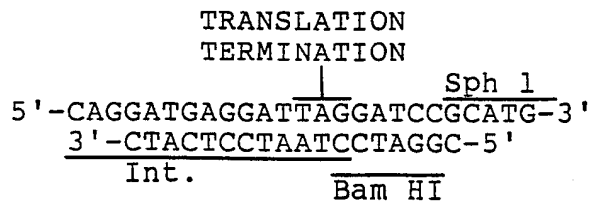
Using standard DNA synthesizing techniques, the following double stranded linker was synthesized:



This linker contains a Sac I sticky end followed by Bam HI plus nine nucleotides of the putative ribosome binding site (partially overlapping with the Bam HI site) in front of the initial 15 nucleotides of the protease (Prt) coding sequence. The 30 nucleotide complementary sequence starts with the 3' Sac I sticky end which extends to the fourth nucleotide from the 3' end of the first strand leaving a three nucleotide single strand tail at the 3' end.

This double stranded linker was ligated to the linearized plasmid and was used to modify the upstream (5') sequences of the pol gene, using the same crossover linker mutagenesis method as described for the rev gene in Example 1 and the vif gene in Example 2, to delete some of the 5' non-coding flanking sequences plus some coding sequences of the protease gene.

To modify the downstream sequences, the following oligonucleotide linker was synthesized:



This linker contained fifteen nucleotide overlapping
 5 sequences of the integrase gene (Int.) which include the
 termination codon of translation followed by Bam HI and an
 Sph I sticky end at the 3' end. The 18 nucleotide
 complementary sequence was used to protect the Bam HI site.

By employing the crossover linker mutagenesis as
 10 described in Examples 1 and 2, the linker was used to
 delete the 3' non-coding sequences and to add a Bam HI
 restriction site.

The resulting pUC 18-pol plasmid, containing no
 non-coding flanking sequences at either end, was digested
 15 with Bam HI and the Bam HI fragment was isolated and
 inserted into the Bam HI site of pACYM1 in the correct
 orientation to form pACYM1-pol.

The pACYM1-pol DNA was used to co-transfect Spodotera
frugiperda (SF9) cells with wild type AcNPV DNA to isolate
 20 a recombinant baculovirus AcNPV-HIVYKpol virus.

The recombinant AcNPV-HIVYKpol virus was used to
 infect SF9 cells to express the pol gene. The AcNPV-HIVYK
 pol virus infected SF9 cells were harvested at 48, 72, 96
 and 120 hours after infection and the total cellular
 25 proteins were subjected to polyacrylamide gel electro-
 phoresis with Coomassie blue staining. The results are
 shown in Fig. 4 in which lane 2 shows the 48 hour product,
 lane 3 shows the 72 hour product, lane 4 shows the 96 hour
 30 product and lane 5 shows the 120 hour product. Lane 6
 shows the wild type AcNPV infected SF9 cells with
 polyhedrin protein (p), lane 7 represents the uninfected
 SF9 cells and lane 1 shows the molecular weight markers.

An approximately 95k Dalton pol protein (as shown with an arrow) was synthesized and accumulated in virus infected cells, representing approximately 30% of the total cellular protein.

- 5 The AcNPV-HIVYKpol virus was deposited at the American Type Culture Collection of 12301 Parklawn Drive, Rockville, MD 20852, USA on November 30, 1988 under the terms of the Budapest Treaty and is identified by the deposit No. ATCC VR 2233.

10 EXAMPLE 4 - ANTIBODIES AGAINST VIF PROTEIN DURING AIDS
 INFECTION

- Antibodies against vif and rev proteins in several individuals having HIV-1 infection were analyzed using the vif and rev proteins expressed in SF9 cells infected with
15 recombinant baculoviruses carrying either the full length vif or rev gene of HIV-1 under the control of the polyhedrin gene promoter.

- The level of intracellular vif protein expression in the recombinant infected SF9 cells at 72 hours of
20 infection was approximately 25% of the total cellular protein. The level of intracellular rev protein in recombinant baculovirus infected SF9 cells after 72 hours of infection was approximately 15% of the total cellular protein. These two proteins were readily visible after
25 staining the gels with Coomassie Blue.

- The SF9 cellular proteins, following recombinant baculovirus infection, were electrophoresed in polyacrylamide gels containing sodium dodecyl sulfate (SDS-PAGE), transferred to nitrocellulose filters and
30 incubated with HIV-1 infected human sera collected from patients at different clinical stages. Figure 6 shows a typical Western blot of vif and rev proteins. The reaction was completed after washing and incubations with HRP-conjugated anti-species antibody and precipitable
35 substrate (4-chloro-1-naphthol). Lanes 1 and 3 are wild type baculovirus (AcNPV) infected SF9 cell extracts, lane 2 represents AcNPV-rev infected SF9 cell extracts, and line 4 represents AcNPV-vif infected SF9 cell extracts.

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An immunoreactive band just below the 24K marker is a non-specific protein. The 27K vif protein can react with antibodies in certain sera as could the 20K rev protein.

Using this Western blot analysis, sera were tested
 5 from 52 HIV-1 infected healthy donors (group I CDC classification), from 22 HIV-1 infected healthy donors at risk (group II CDC classification), from 18 ARC patients (group III CDC classification) and from 27 AIDS patients (group IV CDC classification). The results are shown in
 10 Table 1 below:

TABLE 1

Prevalence of antibodies against vif
 and rev proteins in HIV-1 infected
 individuals with different clinical stage

Disease Stage	No. of vif Ab ⁺ /Total No. tested (%)	No. of rev Ab ⁺ /Total No. tested (%)
*Asymptomatic (CDC Group I)	17/52 (32.6)	18/52 (34.6)
*Asymptomatic at risk (CDC Group II)	8/22 (36.3)	9/22 (40.9)
*ARC Patients (CDC Group III)	0/18 (0)	4/18 (22.2)
*AIDS Patients (CDC Group IV)	0/27 (0)	6/27 (22.2)

* HIV-1 positive sera determined by Western blots and gp41 recombinant ELISA

As can be seen from the Table above, the sera of 17 of 52 HIV-1 infected healthy donors and 8 of 22 HIV-1 infected healthy donors at risk were found to have antibodies against the vif protein. None of the 18 ARC patients and none of the 27 AIDS patients had a detectable level of vif antibodies. This indicates that the vif antibody is produced early in HIV-1 infection and disappears as the disease progresses to ARC and AIDS. Accordingly, disappearance of vif antibodies may signal the appearance of the clinical signs of HIV-1 infection, so that vif antibodies may be a prognostic indicator for the progression of AIDS from an asymptomatic stage to full blown AIDS.

In contrast to the vif antibodies, the antibodies against the rev protein were present randomly in all stages of HIV-1 infection. The rev protein cannot therefore be used as a prognostic indicator.

INDUSTRIAL APPLICABILITY

As will be apparent from the above, the present invention can be used as a system for expressing foreign genes at high levels in a eukaryotic cell system, and the resulting proteins can be used for a variety of purposes, e.g. in vaccines and in medical diagnostic and prognostic tests.

Claims:

1. A recombinant baculovirus comprising at least a major part of a polyhedrin gene promoter region; at least a transcription termination sequence of a polyhedrin structural gene; and a foreign structural gene having a translation start codon followed by coding sequences and a translation stop codon, said foreign gene being located between said promoter region and said termination sequence; characterized in that, immediately upstream of said start codon, there is provided at least a part of a putative insect cell ribosome binding site for the polyhedrin gene effective for at least partially overcoming resistance of susceptible insect cells to express said foreign gene at a high level.
2. A recombinant baculovirus according to Claim 1 characterized in that said part of said putative ribosome binding site comprises at least the final four nucleotides of the sequence 5'-ACCTATAAAT-3'.
3. A recombinant baculovirus according to Claim 1 characterized in that said part of said putative ribosome binding site comprises the sequence 5'-CCTATAAAT-3'.
4. A recombinant baculovirus according to Claim 1 characterized in that said part of said putative ribosome binding site comprises the sequence 5'-CTATAAAT-3'.
5. A recombinant baculovirus according to Claim 1, Claim 2, Claim 3 or Claim 4, characterized in that said baculovirus containing a restriction site upstream of said part of said putative ribosome binding site and a restriction site downstream of said foreign gene.
6. A recombinant baculovirus according to Claim 1, Claim 2, Claim 3 or Claim 4 characterized in that said foreign structural gene is a gene encoding the rev protein of HIV-1.
7. A recombinant baculovirus according to Claim 6 which is AcNPV-HIVYKrev identified by the deposit number ATCC VR 2231.

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8. A recombinant baculovirus according to Claim 1, Claim 2, Claim 3 or Claim 4 characterized in that said foreign structural gene is a gene encoding the vif protein of HIV-1.
- 5 9. A recombinant baculovirus according to Claim 8 which is AcNPV-HIVPKvif identified by the deposit number ATCC VR 2235.
10. A recombinant baculovirus according to Claim 1, Claim 2, Claim 3 or Claim 4 characterized in that said foreign structural gene is a gene encoding the pol protein of HIV-1.
- 10 11. A recombinant baculovirus according to Claim 10 which is AcNPV-HIVYKpol identified by the deposit number ATCC VR 2233.
- 15 12. A recombinant baculovirus according to Claim 1, Claim 2, Claim 3 or Claim 4 characterized in that said foreign structural gene is a gene encoding the tat protein of HIV-1.
13. A recombinant baculovirus according to Claim 12 which is AcNPV-tatYK identified by the deposit number ATCC VR 2206.
- 20 14. A process for producing a recombinant baculovirus containing a foreign gene; characterized by:
- providing said foreign gene having a translation start codon followed by coding sequences and a translation stop codon;
- 25 adding a nucleotide sequence immediately upstream of said start codon, said added nucleotide sequence comprising at least a part of a putative insect cell ribosome binding site for the polyhedrin gene effective for at least partially overcoming resistance of susceptible insect cells to express said foreign gene at a high level;
- 30 introducing said foreign gene and added nucleotide sequence into a baculovirus vector containing at least a major part of a polyhedrin gene promoter region and at least the transcription termination sequence of a poly-
- 35

hedrin structural gene in a position and orientation to come under transcription control of said promoter region;

5 cotransfecting susceptible insect cells with the resulting baculovirus vector DNA and wild type baculovirus genomic DNA; and

isolating recombinant viruses containing said foreign gene and added nucleotide sequence.

15. A process according to Claim 14 characterized in that said foreign gene is isolated from a natural source and
10 has non-coding flanking sequences on the upstream and downstream sides of said foreign gene, and in that at least said upstream non-coding flanking sequences are deleted.

16. A process according to Claim 15 characterized in that
15 said downstream non-coding flanking sequences are also deleted.

17. A process according to Claim 15 characterized in that said upstream flanking sequence is deleted and said nucleotide sequence is added immediately upstream of said
20 translation start codon by producing a crossover linker containing said nucleotide sequence to be added and an homology searching sequence for said foreign gene, ligating said linker to a linearized vector containing said foreign gene and said non-coding upstream flanking
25 sequence, and performing a crossover linker mutagenesis by transfecting competent cells.

18. A process according to Claim 16 characterized in that said downstream non-coding flanking sequences are deleted by producing a crossover linker containing an homology
30 searching sequence for said foreign gene, ligating said linker to a linearized vector containing said foreign gene and non-coding downstream flanking sequences, and performing a crossover linker mutagenesis by transfecting competent cells.

35 19. A process according to Claim 14, Claim 15 or Claim 16 characterized in that said added nucleotide sequence comprises at least the final four nucleotides of the

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sequence 5'-ACCTATAAAT-3'.

20. A process for producing a baculovirus transfer vector suitable for producing a recombinant baculovirus containing a foreign gene, characterized by:

5 providing said foreign gene having a translation start codon followed by coding sequences and a translation stop codon;

adding a nucleotide sequence immediately upstream of said start codon, said added nucleotide sequence comprising at least a part of a putative insect cell ribosome binding site for the polyhedrin gene effective for at least partially overcoming resistance of susceptible insect cells to express said foreign gene at a high level;

10 introducing said foreign gene and added nucleotide sequence into a baculovirus vector containing at least a major part of a polyhedrin gene promoter region and at least the transcription termination sequence of a polyhedrin structural gene in a position and orientation to come under transcription control of said promoter
20 region; and

amplifying the resulting modified baculovirus transfer vector.

21. A baculovirus transfer vector suitable for producing a recombinant baculovirus containing a foreign gene,
25 characterized by:

at least a major part of a polyhedrin gene promoter region; at least a transcription termination sequence of a polyhedrin structural gene; a foreign structural gene having a translation start codon followed by coding
30 sequences and a translation stop codon, said foreign gene being located between said promoter region and said termination sequence; and, immediately upstream of said start codon, at least a part of a putative insect cell ribosome binding site for the polyhedrin gene effective
35 for at least partially overcoming resistance of susceptible insect cells to express said foreign gene at a high level.

22. A process for expressing foreign gene proteins at a high level in a baculovirus expression system, characterized by:

5 infecting susceptible organisms selected from the group consisting of insect cells and insects with a recombinant baculovirus containing: at least a major part of a polyhedrin gene promoter region; at least a transcription termination sequence of a polyhedrin structural gene; a foreign structural gene having a translation start codon followed by coding sequences and a translation stop codon, said foreign gene being located between said promoter region and said termination sequence; and, immediately upstream of said start codon, at least a part of a putative insect cell ribosome binding site for the polyhedrin gene effective for at least partially overcoming any resistance of susceptible insect cells to express said foreign gene at a high level; and
10 extracting said foreign gene protein from said cells or associated fluid after a suitable period of time following said infection.

23. A process according to claim 22 characterized by said foreign gene is a gene encoding the rev protein of HIV-1.

24. A process according to claim 22 characterized by said foreign gene is a gene encoding the vif protein of HIV-1.

25 25. A process according to claim 22 characterized by said foreign gene is a gene encoding the pol protein of HIV-1.

26. A process according to claim 22 characterized by said foreign gene is a gene encoding the tat protein of HIV-1.

27. A foreign gene protein expressed by a process characterized by:

30 infecting susceptible insect cells or insects with a recombinant baculovirus containing: at least part of a polyhedrin gene promoter region; at least a transcription termination sequence of a polyhedrin structural gene;
35 a foreign structural gene having a translation start codon followed by coding sequences and a translation stop codon,

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said foreign gene being located between said promoter region and said termination sequence; and, immediately upstream of said start codon, at least a part of a putative insect cell ribosome binding site for the polyhedrin gene
5 effective for at least partially overcoming any resistance of susceptible insect cells to express said foreign gene at a high level; and

extracting said foreign gene protein from said cells or associated fluid after a suitable period of time
10 following said infection.

28. The rev protein of HIV-1 characterized in that said protein is produced by a process according to claim 23.

29. The vif protein of HIV-1 characterized in that said protein is produced by a process according to claim 24.

15 30. The pol protein of HIV-1 characterized in that said protein is produced by a process according to Claim 25.

31. The tat protein of HIV-1 characterized in that said protein is produced by a process according to Claim 26.

32. A reagent for a medical test characterized in that
20 said reagent comprises a protein as claimed in Claim 27, Claim 28, Claim 29, Claim 30 or Claim 31.

33. A vaccine for enhancing immunity against a disease, characterized in that said vaccine contains a protein as claimed in Claim 27.

25 35. A vaccine for enhancing immunity against acquired immunodeficiency syndrome (AIDS), characterized in that said vaccine contains a protein according to Claim 28, Claim 29, Claim 30 or Claim 31.

36. A prognostic indicator for the progression of HIV
30 infection from an asymptomatic stage to a stage exhibiting symptoms of AIDS, characterized in that said indicator comprises a vif protein of an HIV virus.

37. An indicator according to Claim 36 characterized in that said vif protein is a protein according to Claim 29.

35 38. The use of a prognostic indicator for the progression of HIV infection from an asymptomatic stage to a stage

-29-

exhibiting symptoms of AIDS characterized in that said indicator comprises a vif protein of an HIV virus.

39. The use according to Claim 38 characterized in that said vif protein is a protein according to Claim 29.

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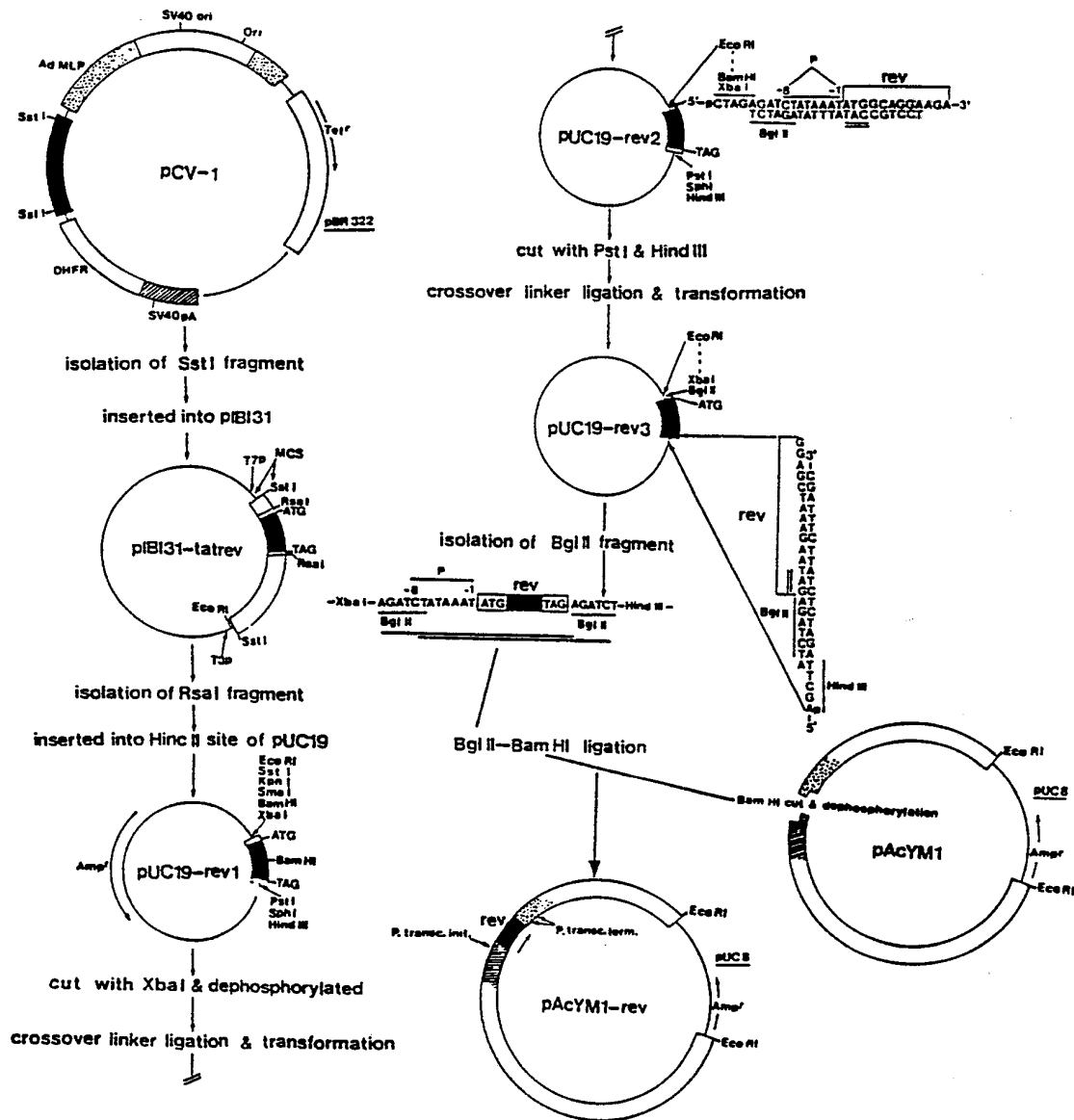
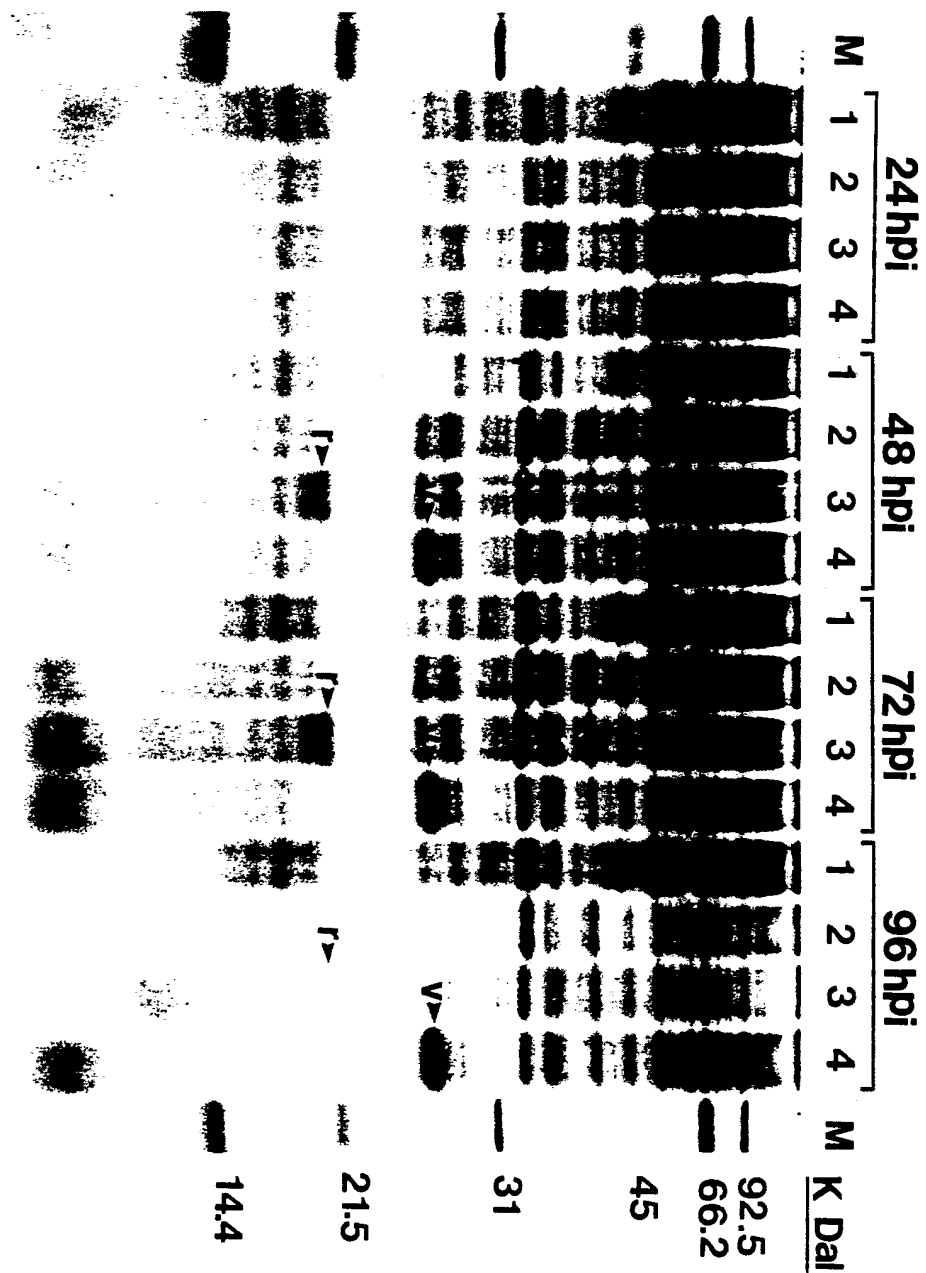


FIG 1

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FIG. 2



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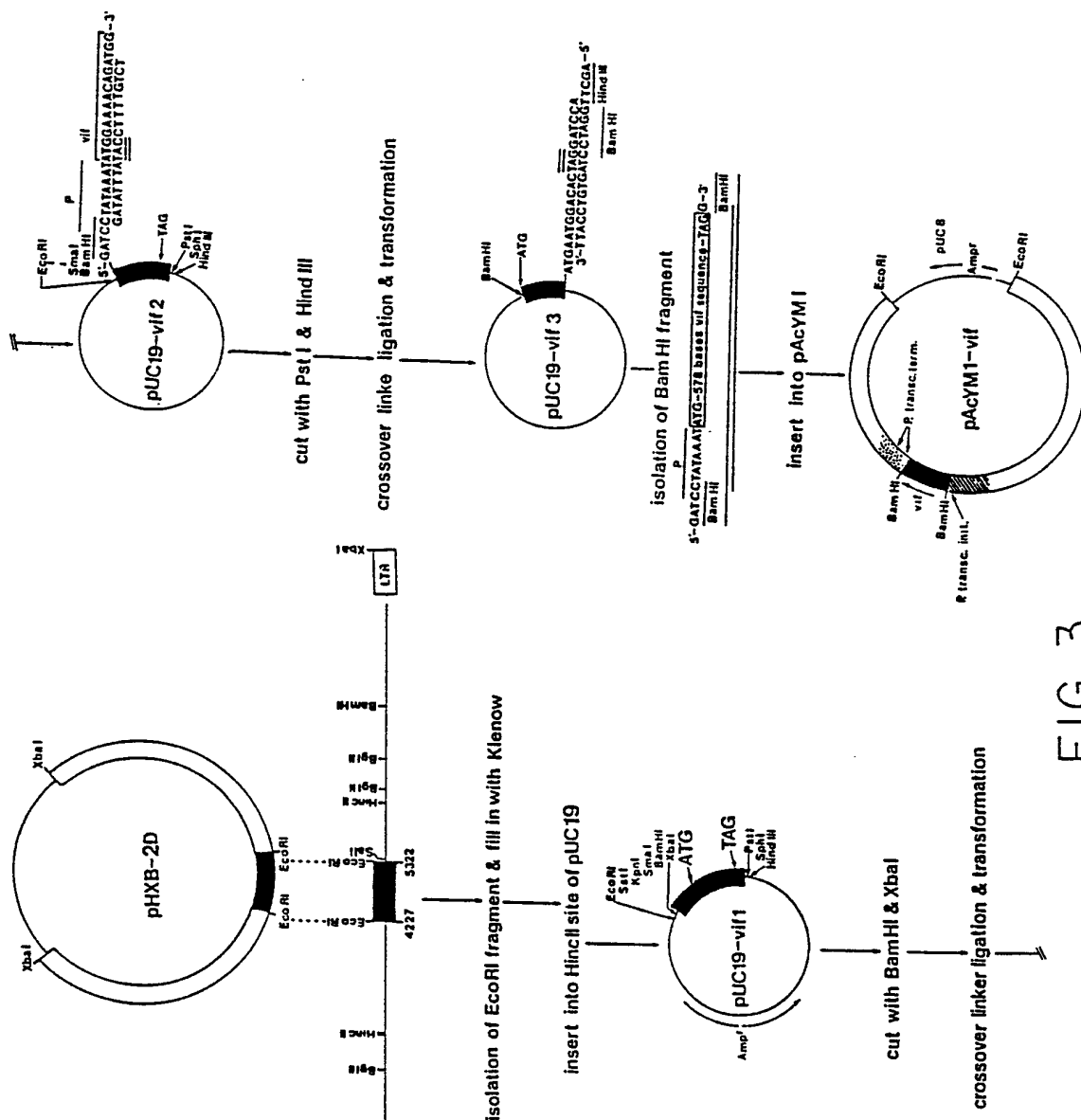
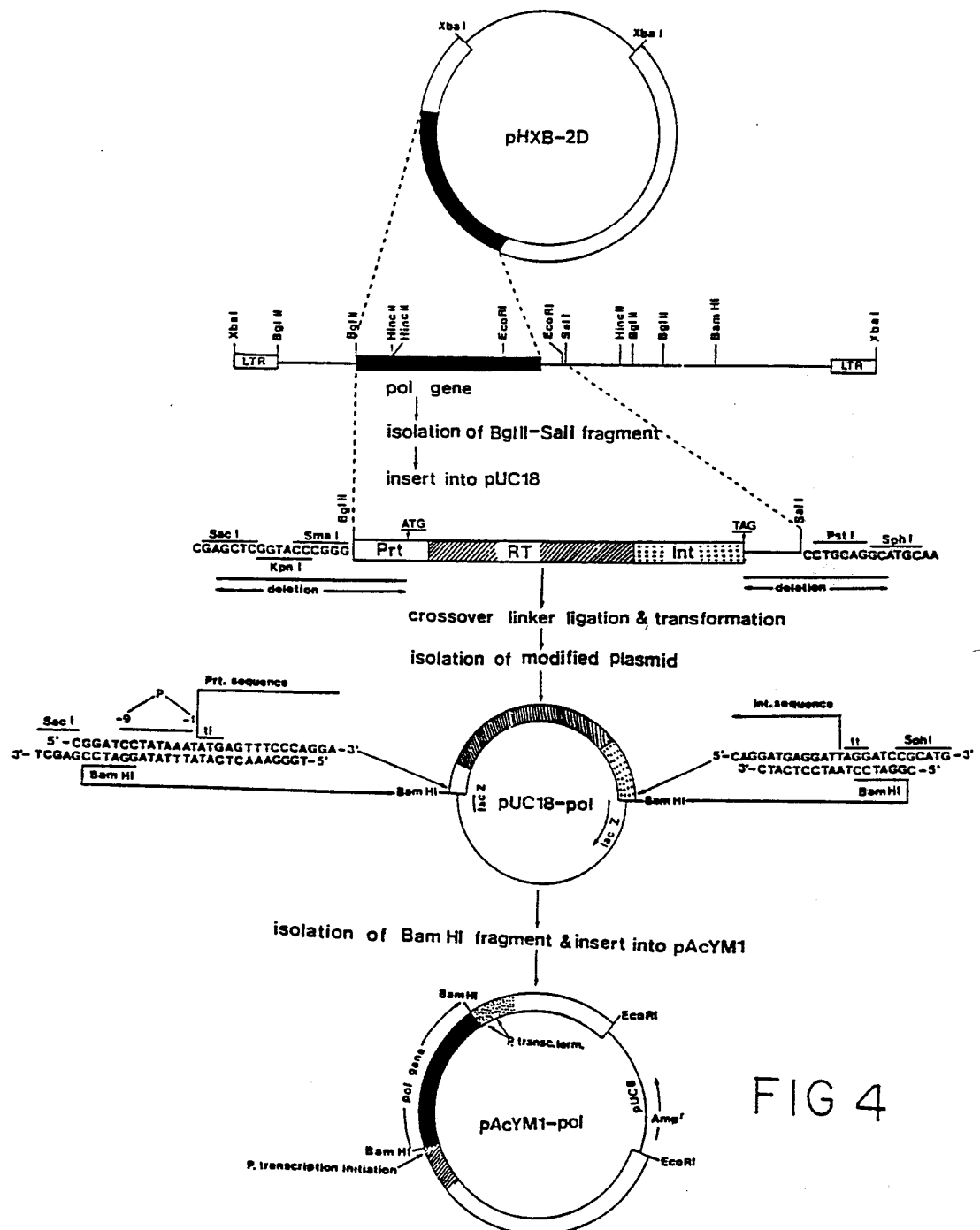


FIG 3

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5/6

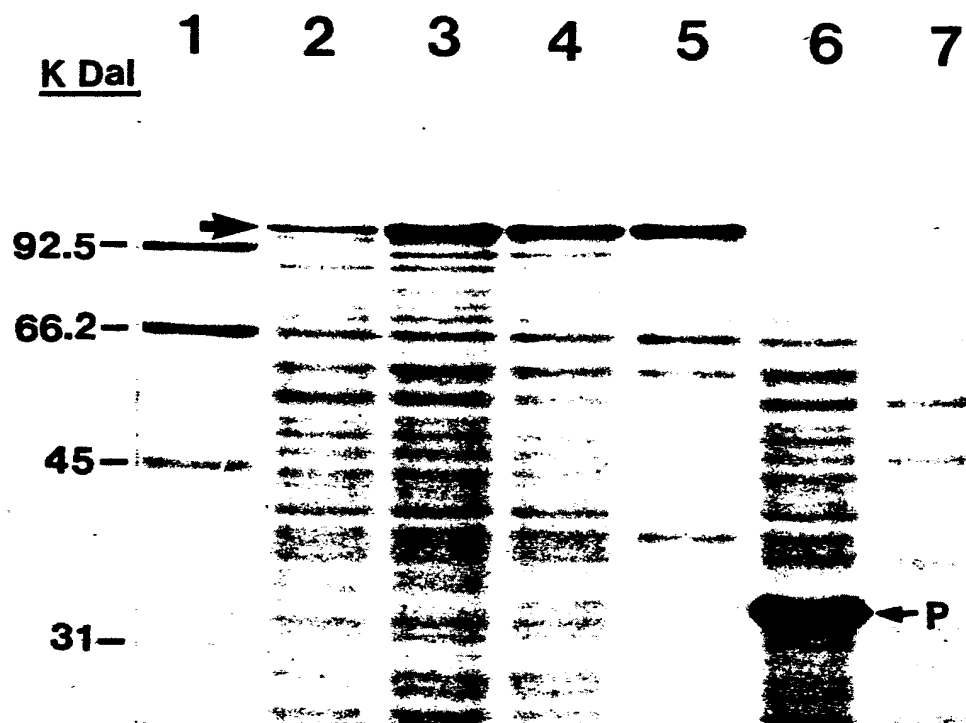


FIG.5

SUBSTITUTE SHEET

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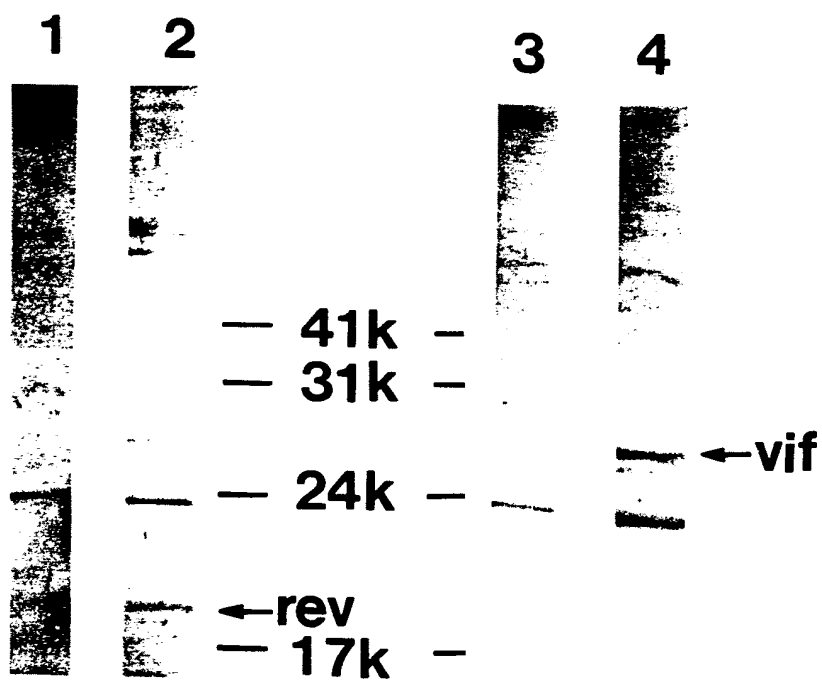


FIG. 6

INTERNATIONAL SEARCH REPORT

International Application No PCT/CA 90/00061

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) * According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁵ : C 12 N 15/86, C 12 P 21/00, C 12 N 15/49, A 61 K 39/21											
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%; border-bottom: 1px solid black;">Classification System</th> <th style="border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="border-right: 1px solid black; padding: 5px;">IPC⁵</td> <td style="padding: 5px;">C 12 N, C 12 P</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸</div>			Classification System	Classification Symbols	IPC ⁵	C 12 N, C 12 P					
Classification System	Classification Symbols										
IPC ⁵	C 12 N, C 12 P										
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border-bottom: 1px solid black;">Category ⁹</th> <th style="width: 70%; border-bottom: 1px solid black;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 20%; border-bottom: 1px solid black;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;"> Agricultural & Biological Chemistry, volume 51, no. 6, June 1987, (Tokyo, JP), T. Horiuchi et al.: "High-level expression of the human-alpha-inter- feron gene through the use of an improved baculovirus vector in the silkworm, Bombyx mori", pages 1573-1580 see figure 1 <div style="text-align: center;">--</div> </td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-5,14-22</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;"> The Journal of General Virology, volume 68, no. 5, May 1987, SGM, (GB), Y. Matsuura et al.: "Baculovirus expression vectors: the requirements for high level expression of proteins, including glycoproteins", pages 1233-1250 see figure 1 <div style="text-align: center;">--</div> <div style="text-align: right;">./.</div> </td> <td></td> </tr> </table>			Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	X	Agricultural & Biological Chemistry, volume 51, no. 6, June 1987, (Tokyo, JP), T. Horiuchi et al.: "High-level expression of the human-alpha-inter- feron gene through the use of an improved baculovirus vector in the silkworm, Bombyx mori", pages 1573-1580 see figure 1 <div style="text-align: center;">--</div>	1-5,14-22	X	The Journal of General Virology, volume 68, no. 5, May 1987, SGM, (GB), Y. Matsuura et al.: "Baculovirus expression vectors: the requirements for high level expression of proteins, including glycoproteins", pages 1233-1250 see figure 1 <div style="text-align: center;">--</div> <div style="text-align: right;">./.</div>	
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>											
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;"> Date of the Actual Completion of the International Search 13th June 1990 </td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;"> Date of Mailing of this International Search Report 11. 07. 90 </td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;"> International Searching Authority EUROPEAN PATENT OFFICE </td> <td style="border-bottom: 1px solid black; padding: 5px;"> Signature of Authorized Officer <div style="text-align: center;"> H. DANIELES </div> </td> </tr> </table>			Date of the Actual Completion of the International Search 13th June 1990	Date of Mailing of this International Search Report 11. 07. 90	International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer <div style="text-align: center;"> H. DANIELES </div>					
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, " with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	EP, A, 0265785 (MICROGENESYS, INC.) 4 May 1988 see page 3, line 15 -----	10,25,30

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

CA 9000061
SA 35053

CA 9000061
SA 35053

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 03/07/90. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0265785	04-05-88	AU-A- 7984287	21-04-88
		JP-A- 63207397	26-08-88
